

AD \_\_\_\_\_

Award Number: DAMD17-01-1-0091

TITLE: Bone Mineral Density, Sex Steroid Genes, Race, and Prostate Cancer Risk

PRINCIPAL INVESTIGATOR: Francesmary Modugno, Ph.D., M.P.H.

CONTRACTING ORGANIZATION: University of Pittsburgh  
Pittsburgh, PA 15260

REPORT DATE: September 2005

TYPE OF REPORT: Annual

**20060223 509**

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved  
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 01-09-2005	2. REPORT TYPE Annual	3. DATES COVERED (From - To) 1 Sep 2004 – 31 Aug 2005			
4. TITLE AND SUBTITLE Bone Mineral Density, Sex Steroid Genes, Race, and Prostate Cancer Risk			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER DAMD17-01-1-0091		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S)  Francesmary Modugno, Ph.D., M.P.H.  E-mail: fm@cs.cmu.edu			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  University of Pittsburgh Pittsburgh, PA 15260			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT <p>The goal of this project is to determine whether bone mineral density (assumed to be an integrated marker of sex steroid hormone exposure) is a risk factor for prostate cancer; and (2) to identify prostate cancer susceptibility alleles among genes in the sex steroid pathway. To address these aims, we are undertaking a case-control study of African American and Caucasian men in Pittsburgh, PA and Birmingham, AL. As of 7/31/04, 163 Caucasian and 19AA cases, 159 Caucasians and 14 AA controls with PSA &lt;3.0 ng/mL frequency matched by age and race to Hip, spine and total body BMD is measured by Dual-energy X-ray Absorptiometry (DXA) on all participants. Blood specimens have been used to isolate DNA on 255 subjects. Polymerase Chain Reaction (PCR) techniques are being used to determine allelic distributions of genotypes for sex steroid metabolism, biosynthesis and action genes. Risk factor data are obtained by an in-person interview and are immediately scanned into the study database. Caucasian recruitment has been completed. AA recruitment is ongoing. Upon completion of AA recruitment and data collection, we will evaluate the role of BMD and candidate genotypes in prostate cancer risk by race. We will further examine the interaction between BMD and genotypes to evaluate the hormonal environment – gene interaction and its effect on prostate cancer risk.</p>					
15. SUBJECT TERMS Prevention, epidemiology, genetic epidemiology, etiology, gene-gene and gene-environment interaction, genetic risk, bone mineral density, sex steroid hormone					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 16	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	19b. TELEPHONE NUMBER (include area code)		

## **Table of Contents**

<b>Cover.....</b>	<b>1</b>
<b>SF 298.....</b>	<b>2</b>
<b>Table of Contents.....</b>	<b>3</b>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>4</b>
<b>Key Research Accomplishments.....</b>	<b>8</b>
<b>Reportable Outcomes.....</b>	<b>8</b>
<b>Conclusions.....</b>	<b>8</b>
<b>References.....</b>	<b>8</b>
<b>Appendices.....</b>	<b>9</b>

**INTRODUCTION:**

The goal of this project is to determine whether bone mineral density (assumed to be an integrated marker of sex steroid hormone exposure) is a risk factor for prostate cancer; and (2) to identify prostate cancer susceptibility alleles among genes in the sex steroid pathway. To address these aims, we are undertaking a case-control study of African American and Caucasian men in Pittsburgh, PA and Birmingham Alabama. Cases are 100-150 African American and 150 Caucasian men with histologically-confirmed prostate cancer. Controls are age and race frequency-matched men who have a prostate specific antigen (PSA) level < 3.0 ng/mL. Hip, spine and total body body mass index (BMD) is measured by Dual-energy X-ray Absorptiometry (DXA). Blood is used to obtain DNA. Polymerase Chain Reaction (PCR) techniques will be used to determine allelic distributions of genotypes for sex steroid metabolism, biosynthesis and action genes. Risk factor data are obtained by an in-person interview. Pathology information will be collected using standardized medical abstraction and all pathology will be confirmed by a central pathologist. Upon completion recruitment and data collection, we will evaluate the role of BMD and candidate genotypes in prostate cancer risk by race. We will further examine the interaction between BMD and genotypes to evaluate the hormonal environment – gene interaction and its effect on prostate cancer risk.

**BODY:**

In this section, we describe our accomplishments according to the Work Plan originally approved:

*Task 3 Recruiting of Subjects and Obtaining of Data, Months 6-30*

Please see tables 1-3 for summaries to 8/31/05. In short, we have recruited 217 Caucasian cases, 209 Caucasian controls, 50 African American (AA) cases and 44 AA controls (plus an additional 20 AA controls whose data have not yet been entered, for a total of 64 AA controls).

*Task 4 Performance of Laboratory Assays, Months 12-31:**a. Isolate DNA from blood samples*

- DNA was isolated on 541 subjects thus far and continues to move forward.
- DNA was quantitated and diluted to 40ng/ul

*b. Assay samples (600) to detect sex steroid related genetic polymorphisms and record results on study forms*

- The following genotyping assays were performed on a subset of specimens:
  - a. AIB1/SRC3 - steroid receptor coactivator 3; CAG (glutamine) repeat polymorphism
  - b. CYP11A - cholesterol side chain cleavage enzyme; pentanucleotide repeat [(TTTA)<sub>n</sub>] in the promoter
  - c. SHBG - pentanucleotide repeat [(TAAAA)<sub>n</sub>] located in an Alu sequence at the 5' boundary of the promoter
  - d. CYP19 - aromatase; intronic tetranucleotide repeat [(TTTA)<sub>n</sub>]
  - e. HSD11B1 - 11-beta hydroxysteroid dehydrogenase; a CA repeat

- We will assay all subjects in batches towards the end of this grant year in order to control for potential batch variability or errors

*Task 5 Data Entry, Months 12-32:*

- a. *Enter, verify and clean interview, anthropometric, physical activity, pathology, DXA, and laboratory assay data via the PoP computerized data entry system*

- As previously reported, we implemented the questionnaire in TeleForm, so that data entry is ongoing. All data is entered when a subject is interviewed. Thus, data on all subjects recruited to date is already in the study database. We have randomly reviewed 10% of the interview forms and compared the data with the database to ensure accuracy.

#### *Task 6 Interim Analyses of Data, Months 18-30 :*

- a. *Perform interim statistical analyses of data periodically*

- See tables 1-3 for data

- a preliminary review of these data suggest that the AA population is less likely to be married and have a lower level of education. This most likely reflects the referral patterns of AA with prostate cancer and the practice from which we recruit our Caucasian subjects (Dr. Joel Nelson, Chair of Urology, University of Pittsburgh Medical Center). These differences will be controlled for in our analyses (by including a variable in our models).

#### **Overall Study Progress:**

To date, we have completed recruitment of Caucasians in Pittsburgh.

A major problem with this study has been the recruitment of African Americans. Our initial minority site, Baltimore, MD, was never approved by the Human Subjects Committee. We then arranged for a second minority site, Alabama. Despite extensive personnel efforts, recruitment in Alabama has been slow (see below). In Pittsburgh, we have tried a variety of mechanisms to increase minority recruitment (see below), which for the most part have had a minimal effect on recruitment. Thus, the only way we will reach our minority recruitment goal is to extend the study, because we have shown slow but consistent recruitment of about 3 AA cases a month study wide.

#### **Pittsburgh Progress:**

We obtained IRB approval from the University of Pittsburgh to commence recruitment of subjects in Pittsburgh. Recruitment began in February 2002. Cases are recruited from all newly diagnosed cases of prostate cancer seen in the practice of Dr. Joel Nelson. Controls are men who have participated in a population-based prostate cancer screening trial (the Prostate, Lung, Colo-rectal and Ovarian (PLCO) Cancer Trial) and are frequency matched to cases by age and race. The summary of recruitment to date is in Table 1. We recruited approximately 4 men per week in Pittsburgh (2 cases, 2 controls), which was in line with the anticipated recruitment schedule. Because interview data is scanned in weekly, we are able to provide interim data analyses. Table 2 summarizes the baseline data on all recruited subjects.

Minority recruitment has been low. To this end, we have tried a variety of techniques to increase enrollment. We have worked with the Center for Minority Health to design targeted recruitment materials (see appendix). We have also worked out a recruitment method with our IRB so that every AA man diagnosed with prostate cancer at a UPMC facility (about 40 men per year) will be informed about the study. To date this had provided a small increase in minority recruitment.

Other approaches we have tried: we have given presentations at urology meetings and prostate cancer support group meetings throughout the city. We have worked with the American Cancer Society and other local

organizations to advertise our study and support recruitment. This has had minimal impact on our recruitment. Most African American men with prostate cancer are seen outside the UPMC health system. We have contacted physicians in the other health system to engage their support of our study, and offered to compensate their staff for time engaged in helping with this study (using monies from Dr. Modugno's R&D fund). The two practices that see most African Americans cases in the area refused to cooperate because they do not wish to collaborate with UPMC investigators. We therefore have exhausted all possibilities for African American recruitment in Pittsburgh.

**Baltimore Progress:**

We were unable to obtain IRB approval from the DOD Human Subjects Committee for the Baltimore site. We have therefore had to drop this site from the study. We invested a great deal of time and effort in trying to launch this study in Baltimore, and are disappointed to not include them in the study because in the preliminary recruitment efforts, approximately 2-3 AA cases *per week* were being referred into the study.

**Alabama Progress:**

We received DOD IRB approval in 2004 to commence minority recruitment at the Alabama site. The site is under the supervision of Dr. James Shikany. During 2004 we worked with the Alabama investigators to revise the Manual of Operations for their site and to put into place all the study procedures, including sending data and specimens to Pittsburgh. We also trained the field interviewer to ensure consistency in questionnaire administration. Concurrently, the Alabama investigative team met with urologists in the area to set up recruitment procedures.

Dr. Shikany and his staff have set up recruitment in several urology clinics in the Birmingham area, including Dr. Urban at the University of Alabama, Dr. Tully (AL Urology Associates) and Dr. Cohn (St. Vincent's Medical Center). They have also arranged for recruitment at the local VA hospital. Over a period of about 8 months of recruitment, they have consented 26 African American cases. Controls have been recruited from healthy men participating at the PLCO trial.

Progress in Alabama has been slower than anticipated. We anticipated recruiting approximately 100 AA cases over a 1-2 year period. Unlike Caucasian recruitment in Pittsburgh, in which the collaborating physicians refer men into the study, the physicians in Alabama require our recruiter to be present in clinic to talk to gentlemen after their doctor visit. This is extremely labor intensive and reduces the number of potential subjects our recruiter can contact per day. In addition, minority men are more reluctant to participate in a study and the no-show rate for AA subjects is high relative to our Caucasian subjects.

We have worked with the site PI to provide additional resources and mechanisms to increase enrollment and we hope that this support will increase AA enrollment over the final study year.

**Exclusion Criteria**

The following are the criteria used to exclude men from participation in this study.

<40 or >80 years of age

Inability to consent to medical procedures.

History of hyper or hypothyroidism, hyperparathyroidism, renal disease, or bone disorders

History of hypogonadism

History of Bone Disease/problems – osteoporosis, Paget's disease, osteomalacia, osteogenesis imperfecta,  
Chronic (>3 months) glucocorticoid therapy  
Use of testosterone supplementation (>3 months)  
Use of bisphosphonate supplementation (>3 months)  
Bilateral hip replacement  
Kidney or liver transplant recipient  
Previous diagnosis of cancer, except basal/squamous cell skin cancer  
trouble absorbing vit D., vit D deficiency, calcium abnormality, brittle bones  
2 or more non-traumatic fractures over a lifetime or 1 or more non-traumatic fracture in the last year.  
For prostate cancer cases, evidence of bone metastases  
For controls, PSA levels above 3 ng/mL within the last 3 months

### Data Collection

The following data are collected on all participants:

- demographics, lifestyle factors and medical history via a ½ hours in-person interview
- hip, spine and total BMD via a DXA scan. Results are abstracted onto a study form
- urine sample (Pittsburgh only)
- 35 ml of blood. This is used to isolate DNA for the current study. In addition, the following specimens are banked:
  - o serum (8x1mL)
  - o plasma (8x1mL)
  - o buffy coat
  - o clot
- height, weight and hip circumference (measured by study personnel during study visit). Results are recorded on a study form.

### Laboratory Assays

All genotyping assays are done in the laboratory of Dr. Robert Ferrell. High molecular weight DNA is extracted from peripheral blood leukocytes by the salting-out procedure. Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism(RFLP) techniques will be used to identify polymorphisms in the sex steroid metabolism pathway. Restriction fragment length polymorphisms are genotyped by amplification of the variable site using unique sequence flanking primers, digestion with an appropriate restriction endonuclease, resolution of the fragments on 2% agarose gels and visualization under ultra violet light after ethidium bromide staining. Single nucleotide polymorphisms that do not alter a restriction site are assayed by a modified allele specific oligonucleotide ligation assay. Length polymorphisms are genotyped by amplification using unique sequence flanking primers, one of which is labeled with a fluorescent dye (FAM, HEX or TET; Research genetics, Huntsville, AL). The products are resolved on the ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA) and the resulting gel images are analyzed using the GENESCAN software package. These protocols are standard in Dr. Ferrell's lab. Genotypes are assigned by two independent readers by directly comparing test samples to sequence-verified control samples run on the same gel. Conflicts are resolved by repeat genotyping.

We have tested the laboratory assays on a sample of specimens early in our recruitment. The assays appear to be working.

### BMD Measurements

Hip, spine and total body BMD will be measured by Dual-energy X-ray Absorptiometry (DXA) using a Hologic QDR-4500A (Hologic, Inc., Waltham, MA) in the Laboratory of Dr. Susan Greenspan. Quality control is assessed by daily quality control scans with the phantom provided by the manufacturer. We will also have a subset of scans (10%) reanalyzed by Synarc, Inc. (Bedford, MA), which provides quality control for large scale studies, including several of Dr. Greenspan's studies. All DXA results are recorded on a standard study form for data entry.

### Problems encountered and measures taken:

Minority recruitment has been very difficult. As discussed above, we have taken several measures in both Pittsburgh and Alabama to try and increase enrollment.

### KEY RESEARCH ACCOMPLISHMENTS:

- completed Caucasian recruitment in Pittsburgh
- all Pittsburgh demographic and BMD data are cleaned and ready to be analyzed
- began minority recruitment in Alabama
- isolated and quantitated DNA from 541 samples to date, with isolation continuing
- genotyped a subset of specimens on 5 sites

### REPORTABLE OUTCOMES:

As stated in the original grant application, we have used this opportunity to collect data and specimens to bank in order to support future studies. For example, we collected urine specimens on men participating at the Pittsburgh site. These specimens were used in an analysis to assess the role of surviving as a marker of prostate cancer risk. The work was accepted for publication in the *Journal of Urology* (see appendix).

### CONCLUSIONS:

We are pleased with our progress in Caucasian recruitment and the laboratory work, and anticipate the successful completion of this project (although it will be late). We recognize that we are behind in AA recruitment, although we have worked to ensure resources will be available to complete this recruitment over the next year. We are working to recruit an additional 50 AA cases with controls in Pittsburgh and Alabama. To ensure that geographical differences between the two sites will not confound any results, we will control for recruitment site in all analyses and will recruit a small number of Caucasian cases and controls in Alabama. We will also perform separate analyses by recruitment site.

### REFERENCES:

None

### APPENDICES:

African American Recruitment Flyer

In Press Manuscript: Benjamin Davies, Joseph Chen, Francesmary **Modugno**, Joel Weissfeld, Doug Landsittel, Rajiv Dhir, Joel Nelson, and Robert H. Getzenberg. Contribution of the Prostate Limits the Utility of Survivin in the Detection of Bladder Cancer. To appear in *Journal of Urology*

Table 1: Summary of Recruitment through 8/31/05

Age Range	Caucasian		African-American	
	Cases	Controls	Cases	Controls
40-44	3	3	1	5
45-49	4	4	4	2
50-54	22	18	5	10
55-59	60	59	14	5
60-64	67	66	12	6
65-69	46	44	5	11
70-74	14	14	7	3
75-79	1	1	2	2
Total	217	209	50	44

Table 2: Summary Demographic Statistics on Cases through 8/31/05

	Cases		UPMC Cases		UAB Cases		Caucasian Cases		AA	
	n=267	%	n=240	%	n=24	%	n=217	%	n=50	%
Age (yrs) mean	60.49		60.45		60.92		60.53		60.34	
Race										
Caucasian	217	81.3	217	90.4						
African-American	50	18.7	23	9.6	24	100.0				
Recruitment Site										
UPMC	240	89.9					217	100.0	23	46.0
VA	3	1.1							3	6.0
UAB	24	9.0							24	48.0
Education										
<8 yrs	2	0.7			2	8.3			2	4.0
8 to 11 yrs	8	3.0	4	1.7	4	16.7	4	1.8	4	8.0
12 yrs or HS	50	18.7	45	18.8	4	16.7	35	16.1	15	30.0
post secondary	11	4.1	10	4.2	1	4.2	8	3.7	3	6.0
some college	39	14.6	31	12.9	6	25.0	25	11.5	14	28.0
college grad	65	24.3	60	25.0	5	20.8	57	26.3	8	16.0
postgraduate	92	34.5	90	37.5	2	8.3	88	40.6	4	8.0
Marital Status										
never married	10	3.7	8	3.3	2	8.3	5	2.3	5	10.0
married	231	86.5	212	88.2	18	75.0	196	90.3	35	70.0
widowed	6	2.2	4	1.7	2	8.3	4	1.8	2	4.0
divorced	13	4.9	10	4.2	1	4.2	8	3.7	5	10.0
separated	7	2.6	6	2.5	1	4.2	4	1.8	3	6.0
BMD (g/cm2)	<b>n</b>	<b>Mean</b>	<b>n</b>	<b>Mean</b>	<b>n</b>	<b>Mean</b>	<b>n</b>	<b>Mean</b>	<b>n</b>	<b>Mean</b>
Hip	266	1.02	239	1.01	24	1.06	216	1.01	50	1.05
Spine Lateral	159	0.792	156	0.794			141	0.787	18	0.832
Spine PA	265	1.081	238	1.075	24	1.147	215	1.076	50	1.102
Total Body	259	1.166	232	1.156	24	1.272	209	1.154	50	1.216
LBM	235	62.01	232	62.11			209	61.92	26	62.77
% Body Fat	258	25.57	232	25.79	23	23.06	209	25.85	49	24.39

Table 3: Summary Demographic Statistics on Controls through 8/31/05

	Controls		UPMC Controls		UAB Controls		Caucasian Controls		AA Controls	
	n=254	%	n=236	%	n=10	%	n=209	%	n=44	%
Age (yrs) mean	60.75		61.17		61.60		61.11		58.95	
Race										
Caucasian	209	82.3	208	88.1						
African-American	44	17.3	27	11.4	10	100.0				
Other	1	0.4	1	0.4						
Recruitment Site										
UPMC	236	92.9					208	99.5	27	61.4
VA	8	3.1					1	0.5	7	15.9
UAB	10	3.9							10	22.7
Education										
<8 yrs	2	0.8	1	0.4	1	10.0	1	0.5	1	2.3
8 to 11 yrs	6	2.4	5	2.1	1	10.0	3	1.4	3	6.8
12 yrs or HS	47	18.5	45	19.1			38	18.2	9	20.5
post secondary	17	6.7	15	6.4			14	6.7	3	6.8
some college	54	21.3	48	20.3	3	30.0	42	20.1	12	27.3
college grad	52	20.5	47	19.9	4	40.0	41	19.6	10	22.7
postgraduate	76	29.9	75	31.8	1	10.0	70	33.5	6	13.6
Marital Status										
never married	20	7.9	18	7.6			14	6.7	6	13.6
married	185	72.8	174	73.7	6	60.0	159	76.1	25	56.8
widowed	13	5.1	12	5.1	1	10.0	11	5.3	2	4.5
divorced	31	12.2	28	11.9	2	20.0	22	10.5	9	20.5
separated	5	2.0	4	1.7	1	10.0	3	1.4	2	4.5
BMD (g/cm2)	<b>n</b>	<b>Mean</b>	<b>n</b>	<b>Mean</b>	<b>n</b>	<b>Mean</b>	<b>n</b>	<b>Mean</b>	<b>n</b>	<b>Mean</b>
Hip	254	1.03	236	1.03	10	1.08	209	1.03	44	1.06
Spine Lateral	113	0.800	105	0.794			105	0.794	8	0.876
Spine PA	254	1.095	236	1.096	10	1.071	209	1.097	44	1.085
Total Body	252	1.174	234	1.170	10	1.247	208	1.169	43	1.197
LBM	242	63.18	234	63.03			208	63.10	33	63.48
% Body Fat	252	26.09	234	26.03	10	25.04	208	26.13	43	25.91

# Do You Know that African-American Men Have the Highest Rate of Prostate Cancer in the World ...

**YOU CAN HELP!**

*We Want to find ANSWERS about Prostate Cancer.*

Are you a man  
between ages 40-80  
diagnosed with prostate cancer  
within the last 9 months?



You may be eligible to take part in this study.  
The study takes only ONE visit (about 1 - 2 hours)  
If eligible and you complete the study you will receive:

- \$25
- Parking and Breakfast at no cost to you
- A bone strength test at no cost to you
- A bone health assessment at no cost to you

## Cancer And Prostate Study

For further information and to learn if you may be eligible, call:

**Claudia Leiras (412) 383-2649**

THIS RESEARCH  
STUDY  
IS BEING  
CONDUCTED BY  
GRADUATE SCHOOL OF  
PUBLIC HEALTH  
UNIVERSITY OF PITTSBURGH

## CONTRIBUTION OF THE PROSTATE LIMITS THE USEFULNESS OF SURVIVIN FOR THE DETECTION OF BLADDER CANCER

BENJAMIN DAVIES,\* JOSEPH CHEN, FRANCESMARY MODUGNO, JOEL WEISSFELD,  
DOUG LANDSITTEL, RAJIV DHIR, JOEL NELSON AND ROBERT H. GETZENBERG

*From the Departments of Urology (BD, JC, JN, RHG), Epidemiology (FM, JW), Biostatistics (DL), Pathology (RD, RHG) and Pharmacology (RHG), University of Pittsburgh, Pittsburgh, Pennsylvania*

### ABSTRACT

**Purpose:** We determined if urinary survivin is influenced by the prostate and analyzed survivin levels in a healthy control population.

**Materials and Methods:** This was a blinded, retrospective analysis of frozen urine samples. Three groups were designated. Group 1 patients were diagnosed with prostate cancer and samples were collected immediately prior to radical retropubic prostatectomy. Group 2 patients had already undergone radical retropubic prostatectomy at least 3 months prior to providing a urine sample. Group 3 comprised healthy controls. Groups 1 and 2 patients were recruited at an institutional practice. Group 3 patients were recruited from the local community.

**Results:** The frequency of high survivin scores was much lower in patients who did not have a prostate ( $p < 0.0002$ ). The survivin score was 2 in 35% and 44% of the patients in groups 1 and 3, respectively, while only 9% of those in group 2 had a survivin score of 2 ( $p < 0.0002$ ). Of healthy controls 50% had a false-positive survivin score. Urinary survivin predicted prostate cancer poorly with 52% sensitivity and 50% specificity, and it did not predict any biological features of prostate cancer.

**Conclusions:** Urinary survivin markedly decreases after prostatectomy. There is a 50% false-positive rate when using urinary survivin in controls. These 2 features make urinary survivin a poor bladder cancer biomarker.

**KEY WORDS:** prostate; prostatectomy; prostatic neoplasms; tumor markers, biological; survivin protein, human

Survivin, a member of the inhibitor of apoptosis gene family, was first described as a urinary biomarker for bladder cancer by Smith et al.<sup>1</sup> This report was heralded as a breakthrough in the diagnosis of bladder cancer since it appeared that the test had 100% sensitivity and 95% specificity. Subsequent reports supported these findings, showing urinary survivin detection at the initial diagnosis of bladder cancer and in patients with recurrent bladder cancer after bacillus Calmette-Guerin treatment.<sup>2,3</sup> These studies indicated varying sensitivities and specificities with the most recent study showing 64% sensitivity and 93% specificity.<sup>2</sup>

The worldwide bladder cancer incidence is more than 300,000 and more than 100,000 individuals die of the disease annually.<sup>4</sup> Early detection could decrease the morbidity and mortality of bladder cancer as well as ameliorate the economic and social burden of a lifetime of surveillance and treatment for the disease.<sup>5</sup> Bladder cancer on a cost per patient basis is the most expensive cancer from diagnosis to death.<sup>6</sup>

Tissue based survivin expression has been associated with aggressive cancer behavior and resistance to pharmacotherapy in many malignancies, including transitional cell carcinoma of the bladder, adenocarcinoma of the prostate and renal cell carcinoma.<sup>7</sup> Evidence suggests that survivin is differentially expressed in cancer cells vs normal differentiated tissues.<sup>7</sup> Furthermore, it is the fourth most common

transcriptome up-regulated in human tumors compared to normal tissue.<sup>8</sup>

Studies have shown that the detection of survivin in prostate cancer specimens is a predictor of biological aggressive behavior and biochemical failure, as evidenced by up-regulated mRNA expression or immunohistochemical staining.<sup>9,10</sup> However, survivin expression is reported to be measurable in 38% to 58% of normal prostate tissue, albeit in lower amounts.

As a result of those findings, we hypothesized that urinary survivin could potentially have 3 sources, that is normal prostate, prostate cancer or transitional cell carcinoma of the bladder. Furthermore, if the prostate was a source of urinary survivin, we hypothesized that it could serve as a potential biomarker for prostate cancer.

To test our hypothesis we analyzed urine samples from 3 groups of patients. Group 1 patients were diagnosed with clinically localized prostate cancer. Urine specimens were obtained in the operating room prior to radical retropubic prostatectomy (RRP). Group 2 patients had already undergone RRP at least 6 prior to providing urine and they had no evidence of metastatic disease. Group 3 were frequency matched controls with prostate specific antigen (PSA) less than 3 ng/ml, negative digital rectal examination and no history of cancer or kidney disease.

### PATIENTS AND METHODS

**Patient population.** The University of Pittsburgh institutional review board approved this study. All patients signed an approved consent form. Group 1 patients had clinically localized prostate cancer (cT1c-T3cNxM0) and were recruited at the time of the initial consultation at the urologist office prior to prostatectomy. Urine specimens were obtained in the

Submitted for publication February 5, 2005.

Study received University of Pittsburgh institutional review board approval.

Supported by National Institutes of Health K07-CA80668 (FM) and DAMD 17-01-1-0091 (FM, JW).

\* Correspondence: Department of Urology, University of Pittsburgh, 5200 Centre Ave., Suite 209, Pittsburgh, Pennsylvania 15232 (e-mail: daviesb@upmc.edu).

## PROSTATE LIMITS SURVIVIN USEFULNESS IN BLADDER CANCER

operating room prior to RRP. Group 2 patients were similarly recruited but they had already undergone prostatectomy and they provided urine at least 6 months after RRP. Group 3 patients were recruited from the local community as part of a prostate cancer screening program. They were frequency matched controls by age and race with PSA less than 3 ng/ml, negative rectal examination, no cancer history and no kidney disease. Followup history was unavailable in 2 patients in group 1 and 3 in group 2. A total of 203 patients were included in the study.

**Urinary specimens.** A total of 203 urine specimens were collected at Montefiore and Shadyside Hospital at University of Pittsburgh Medical Center. Straight urine samples were stored at -20°C on the day that they were provided. On the day of analysis they were thawed at room temperature.

**Urine detection of survivin.** Urine specimens were centrifuged at 2,000 RPM for 10 minutes on the day of testing. We performed the assay as described by Smith et al<sup>1</sup> with some minor modifications. We used a 96-well Bio-Dot apparatus (BioRad Laboratories, Hercules, California) to filter 300 µl urine onto polyvinylidene fluoride Immobilon-P™ membrane. The membrane was then blocked with a 5% dried milk and phosphate buffered saline (PBS) solution for 6 hours at room temperature. The positive control consisted of C-terminal recombinant survivin peptide (Abcam, Cambridge, Massachusetts) at increasing concentrations (0.05 to 1 µg/ml). Following 3 washes with 0.25% PBS-Tween 20 the membrane was incubated overnight with polyclonal survivin antibody (Abcam), 1:1,000 dilution, in a 2% dried milk-PBS solution. The next morning the membrane was washed 3 times and horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Amersham Biotech, Piscataway, New Jersey), 1:50,000 dilution, was added in a 2% dried milk-PBS solution. After washing survivin detection was performed using enhanced chemiluminescence.

**Survivin scores.** Bands were quantitated by densitometry. The survivin score was calculated using the scale, 0—undetectable, 1—0.05 to 0.25 µg/ml, 2—0.25 to 1 µg/ml and 3—greater than 1 µg/ml.

**Postoperative followup.** Patients who underwent RRP (groups 1 and 2) had a minimum 6-month followup. PSA determinations at 1, 3 and 6-month intervals were recorded. Pathological examination and relevant medical history were also recorded. Biochemical progression was defined as a sustained increase on 2 or more occasions of PSA greater than 0.2 ng/ml.

**Statistics.** Differences in categorical variables were assessed via the chi-square test or in cases with an expected count of less than 5 with Fisher's exact test. Differences in continuous variables were assessed via the Wilcoxon rank sum test, ie the nonparametric t test, or for testing differences across 3 groups the Kruskal-Wallis nonparametric

ANOVA test. The significance of urinary survivin for predicting prostate cancer features was evaluated in group 1 using the likelihood ratio test from the age adjusted logistic regression model. Models were fit for the outcomes of T stage and extracapsular extension. There were insufficient events to fit statistical models to other prostate cancer features, eg high Gleason score, seminal vesicle invasion, etc.

## RESULTS

**Baseline characteristics among groups.** Table 1 shows each of the 3 groups analyzed. There was no statistical difference between groups 1 and 2 with regard to patient age, preoperative PSA, Gleason score, tumor volume, extracapsular penetration, seminal vesicle involvement, biochemical recurrence, history of cancer or kidney disease.

**Distribution of urinary survivin scores in groups 1 to 3.** Table 2 and the figure show the distribution of survivin by group. Results indicated that the frequency of survivin scores was fairly similar between groups 1 and 3 (control) but group 2 clearly had a lower frequency of high survivin scores. The survivin score was 2 in 35% and 44% of groups 1 and 3, respectively, while only 9% of group 2 had a survivin score of 2. There was a statistically significant association between the frequency of survivin score 2 between groups 1 and 2, and 3 and 2 (each chi-square test p <0.0002).

**Relationship between prostate cancer features and urinary survivin.** Table 1 shows the ability of survivin to predict the clinical features of prostate cancer, namely T stage, extracapsular extension, Gleason score, biochemical progression and tumor volume. In no cases were any of the features predictive. Overall prostate cancer sensitivity and specificity were 52% and 50%, respectively (table 3).

T1

F1

T2

T3

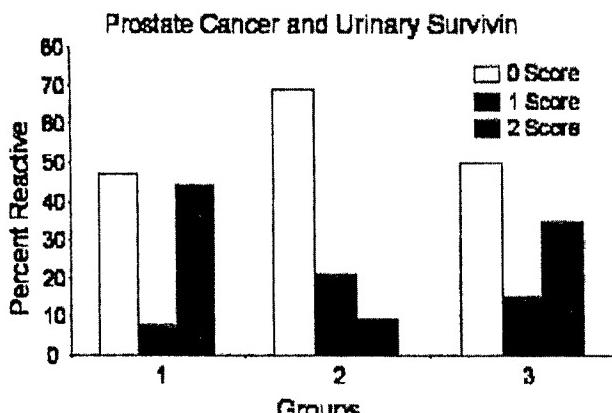
## DISCUSSION

The initial study of urinary survivin was encouraging, showing 95% sensitivity and 100% specificity.<sup>1</sup> As a result, several large clinical trials considered survivin as the principal biomarker for bladder cancer. However, to our knowledge this is the first study to analyze urinary survivin in a healthy cohort of older men with and without prostate cancer. Our study shows a false-positive rate of 50% in the control population.

We also observed that the prostate was the likely source of high urinary survivin scores in our control and prostate cancer groups. Of Group 2, that is patients without a prostate, only 9% had a survivin score of 2, whereas 44% of group 1 patients had a survivin score of 2, strongly suggesting that the prostate secretes survivin into the urine in many patients. There appeared to be a background positive rate (score 1) across all groups. We suspect that this background

TABLE 1. Baseline group characteristics

	Group 1	Group 2	Group 3	p Value
No. pts	78	65	60	
Mean age ± SD	60 ± 6.6	61 ± 6.2	62 ± 3.3	0.27 (Kruskal-Wallis ANOVA)
Mean preop PSA (ng/ml)	6.2	6.6	Less than 3	0.5 (rank sum test group 1 vs 2)
No. Gleason score (%):				
6–7	75 (96)	63 (97)	Not applicable	1.0 (Fisher's exact test group 1 vs 2)
8–9	3 (4)	2 (3)	Not applicable	1.0 (Fisher's exact test group 1 vs 2)
No. pathological T stage (%):				
2a–2c	59 (76)	55 (84)	Not applicable	0.26 (chi-square test group 1 vs 2)
3a–3c	19 (24)	10 (15)	Not applicable	0.26 (chi-square test group 1 vs 2)
No. extracapsular extension (%)	16 (20)	10 (16)	Not applicable	0.7 (chi-square test group 1 vs 2)
No. seminal vesicle invasion	2	0	Not applicable	0.5 (Fisher's exact test group 1 vs 2)
+ surgical margins	3	3	Not applicable	1.0 (Fisher's exact test group 1 vs 2)
No. biochemical progression	2	2	Not applicable	1.0 (Fisher's exact test group 1 vs 2)
Av tumor vol (cm <sup>3</sup> )	1.3	1.2	Not applicable	0.4 (rank sum test group 1 vs 2)
No. Ca history	1	1	0	1.0 (Fisher's exact test group 1 vs 2)
No. kidney disease	0	1	0	1.0 (Fisher's exact test group 1 vs 2)



Prostate cancer and urinary survivin.

TABLE 2. Survivin score by group

Score	Group 1	Group 2	Group 3
No. pts	78	65	60
No. 0 (%)	37 (47)	45 (69)	30 (50)
No. 1 (%)	7 (8)	14 (21)	9 (15)
No. 2 (%)	34 (44)	6 (9)	21 (35)
Av ± SD	0.96 ± 0.95	0.4 ± 0.65	0.85 ± 0.91

TABLE 3. Prostate cancer urinary survivin sensitivity and specificity

Score	% Sensitivity	% Specificity
1	16	23
2	47	41
Overall	52	50

rate could originate from normal renal parenchyma or normal transitional cell epithelium.

Our study is a shift in the clinical application and understanding of urinary survivin. Before to this report no investigators to our knowledge had reported levels of urinary survivin in any patient other than those with bladder cancer. In the initial report of urinary survivin 19 patients with prostate cancer were negative for urinary survivin.<sup>1</sup> However, that report does not indicate if patients were under treatment or when they provided urine. Interestingly the group reported a positive score in a patient with increasing PSA. Other studies of the effect of bacillus Calmette-Guerin treatment, and the relationship of urinary survivin to nuclear matrix protein-22 and urine cytology also did not compare controls to patients with prostate cancer.<sup>2,3</sup>

The positive score of 50% in our control group might appear high, particularly compared to prior reports. We believe that this was a result of our use of a strictly matched control patient population. In prior reports age ranges in the control population were widely scattered and low in number. Smith et al had only 17 healthy patients in their healthy cohort group and mean age ± SD was 47 ± 20.8 years. In the report of Shariat et al the control group had a median age of 68 years but with a range of 21 to 86 years.<sup>2</sup> Unfortunately their healthy control group included only 8 individuals.

There are several limitations to our study. Of our patients 98% were white because of the demographic profile of the average patient at participating urologist practices. Therefore, racial differences in the protein concentrations of urinary survivin were not investigated.

Although no patients in our study had significant kidney disease, we did not collect data on individual creatinine clear-

TABLE 4. Urinary survivin protein concentration, density and grade

Protein Concentration ( $\mu\text{g}/\text{ml}$ )	Density (optical density/mm <sup>2</sup> )	Grade
Less than 0.03	Less than 7	0
0.03–0.1	7–20	1
0.1–0.5	20–200	2
Greater than 0.5	Greater than 200	3

ance, nor did we record individual serum creatinine. As a result, significant differences in creatinine clearance could exist among our study groups. While it is possible, it appears to be a remote confounder, given the size of the study population and the lack of kidney disease in each study group. Moreover, in theory survivin levels should not be influenced by changes in glomerular protein since the source is urothelial in origin. Furthermore, to our knowledge no prior reports show creatinine clearance with relation to urinary survivin.

While reports of urinary survivin have generally followed the same protocol for analysis as the first report,<sup>1</sup> there are neither standardized reagents nor standardized antibodies. All prior reports, including ours, used a polyclonal antibody to human survivin. Not all prior reports explained the provenance of the antibody and, therefore, we assumed that each researcher obtained antibody from different companies. As a result, the antibody specificity of each research group is likely a variable, which could explain some of the reason that survivin studies have disparate results.

The grading system for survivin was started in the original report of Smith et al.<sup>1</sup> It is a product of the lack of linearity between increasing densities of the dot-blot technique and urinary protein concentrations. Table 4 lists the corresponding optical density and protein concentration range for the given grades. The inability to generate a standard curve for the assay introduces another area of concern for this assay.

Another limitation to our study is the disease status of our controls. While we state that they were healthy, it is possible that a proportion may have had quiescent bladder cancer. None of our healthy controls underwent cystoscopy. We believe that this is a remote confounder since the incidence of bladder cancer in the general population is around 0.25%.<sup>4</sup>

#### CONCLUSIONS

The decrease in high urinary survivin after prostatectomy suggests that the prostate makes a significant contribution to urinary levels. The biological features of prostate cancer are not predicted by survivin grade and there is a 50% false-positive rate when using urinary survivin in a healthy control population. All of these features make urinary survivin a poor bladder cancer biomarker.

#### REFERENCES

- Smith, S. D., Wheeler, M. A., Plescia, J., Colberg, J. W., Weiss, R. M. and Altieri, D. C.: Urine detection of survivin and diagnosis of bladder cancer. *JAMA*, **285**: 324, 2001
- Shariat, S. F., Casella, R., Khoddami, S. M., Hernandez, G., Sulser, T., Gasser, T. C. et al: Urine detection of survivin is a sensitive marker for the noninvasive diagnosis of bladder cancer. *J Urol*, **171**: 626, 2004
- Hausladen, D. A., Wheeler, M. A., Altieri, D. C., Colberg, J. W. and Weiss, R. M.: Effect of intravesical treatment of transitional cell carcinoma with bacillus Calmette-Guerin and mitomycin C on urinary survivin levels and outcome. *J Urol*, **170**: 230, 2003
- Parkin, D. M., Bray, F., Ferlay, J. and Pisani, P.: Estimating the world cancer burden: Globocan 2000. *Int J Cancer*, **94**: 153, 2001

PROSTATE LIMITS SURVIVIN USEFULNESS IN BLADDER CANCER

5. Botteman, M. F., Pashos, C. L., Hauser, R. S., Laskin, B. L. and Redaelli, A.: Quality of life aspects of bladder cancer: a review of the literature. *Qual Life Res*, **12**: 675, 2003
6. Botteman, M. F., Pashos, C. L., Redaelli, A., Laskin, B. and Hauser, R.: The health economics of bladder cancer: a comprehensive review of the published literature. *Pharmacoeconomics*, **21**: 1315, 2003
7. Li, F.: Survivin study: what is the next wave? *J Cell Physiol*, **197**: 8, 2003
8. Velculescu, V. E., Madden, S. L., Zhang, L., Lash, A. E., Yu, J., Rago, C. et al: Analysis of human transcriptomes. *Nat Genet*, **23**: 387, 1999
9. Shariat, S. F., Lotan, Y., Saboorian, H., Khoddami, S. M., Roehrborn, C. G., Slawin, K. M. et al: Survivin expression is associated with features of biologically aggressive prostate carcinoma. *Cancer*, **100**: 751, 2004
10. Kishi, H., Igawa, M., Kikuno, N., Yoshino, T., Urakami, S. and Shiina, H.: Expression of the survivin gene in prostate cancer: correlation with clinicopathological characteristics, proliferative activity and apoptosis. *J Urol*, **171**: 1855, 2004